

3rd Annual Citrus Health Research Forum

August 27-30, 2012
Ft. Collins, CO

Speaker and Poster Abstracts

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Speaker Abstracts

Technology for modifying BT toxins against specific targeted pest – use of BT for aphid control (ultimately through transgenic plants) and the steps required to instigate a program for ACP

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Outcome Group 2

Although toxins derived from the bacterium *Bacillus thuringiensis* (Bt) have been used successfully for management of some insect pests, Bt toxins are not effective against the agriculturally important Hemiptera. These sap-sucking insects currently present one of the biggest challenges for agricultural pest management, causing both direct damage and serving as vectors for plant disease. Mutagenesis of Bt toxins can be used to artificially alter toxins for certain properties for enhanced toxicity and we have identified a novel strategy to increase toxin action against aphids. Aphid gut membrane binding property of the Bt toxin was targeted in the toxin modification strategy for increased toxin binding to the pea aphid gut. Pea aphid gut binding peptide (GBP) isolated from peptide display phage library screen was used to modify Cyt2Aa for increased binding to aphid gut. GBP was incorporated in the surface exposed toxin loops and two sets of mutant toxins were prepared. In the first set, GBP sequence was added to the six selected loops and in the second set, six native toxin loops were replaced by GBP sequence. Purified mutant toxins were tested for relative proteolytic stability, binding to insect gut membrane, toxicity to aphids and toxin impact on aphid gut membrane. Eleven out of 12 mutants were proteolytically stable. Although all the mutants exhibited relatively higher binding to aphid gut membrane, only five mutants showed significantly enhanced aphid toxicity. Non-toxicity of seven mutants was attributed to the loss of functional activity of Cyt2Aa. Enhanced aphid mortality due to mutant toxins was caused due to the extensive damage to the pea aphid gut microvilli and gut epithelial cells. This approach shows promise for production of aphid resistant transgenic plants. Selection of a Bt toxin with basal level of toxicity against target pests is a prerequisite for this strategy. Hence while implementing similar program for Asian Citrus Psyllid (ACP), selection of such Bt toxin will indicate that required mechanism is present in ACP. Isolation of ACP gut binding peptide and characterization for gut binding strength and specificity is also an important step. Considering our success with gut binding peptide mediated Bt toxin modification strategy against pea aphid and similarity in the feeding biology of these insects, this approach has potential to develop candidate molecules for transgenic management of ACP.

Host response studies for discovery of pre-symptomatic HLB biomarker and characterization of HLB tolerance/resistance

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Outcome Group 3

Relevance: Host responses to *Liberibacter* infection allow the identification of early (“pre-symptomatic”) RNA/protein/metabolite markers that are manifested long before the visual symptoms of HLB appear enabling the diagnosis of the infection before it is too late. Additionally, the analysis of host responses of citrus cultivars with a varying range of susceptibility to HLB allow the capture of genetic factors that may impart HLB tolerance and/or resistance, which may then be used in transgenic citrus or conventional breeding for disease protection. Thus host response studies have utility both in disease surveillance and management essential for keeping citrus free of ACP and HLB.

Foundation: Functional genomics tools are key to performing host response studies. These tools accurately measure changes in the expression pattern of messenger RNA, small RNA, protein, and metabolites in response to *Liberibacter* infection at different post-exposure times. Analysis of functional genomics data is based upon the comparison with citrus and *Liberibacter* reference genomes. Note that three citrus and three *Liberibacter* genomes have been sequenced with three more in the pipeline to be completed very soon. Existing computational tools have been utilized or new tools have been developed to analyze the transcriptome (messenger and small RNA), proteome, and metabolome data with an ultimate goal of mapping the responsive (RNA/protein/metabolite) markers to specific pathways responsible for plant innate immune response and *Liberibacter*-induced pathogenesis during the early stages of infection. Comparative studies of the *Liberibacter* infection pattern in citrus cultivars with different susceptibilities to HLB are also underway.

Future Directions: Functional genomics tools aid the discovery of pre-symptomatic and HLB tolerance/resistance markers. However, the discovery phase cannot be performed without complete gene and an accurate functional annotation of the citrus genomes, which is yet to be done. Subsequent to the discovery phase, one needs to validate the markers in that they indeed report the pre-symptomatic phase or correspond to HLB tolerance/resistance. A few key studies will be extremely informative in this regard. One may involve the integration of transcriptome, proteome, and metabolome data and construction of the response pathways involving pathogenesis and plant innate immune responses. The other may involve subsequent comparison of the response pathways obtained from the integrated data, which will significantly aid the validation of various markers. Finally, comparison of the response pathways in

psyllid (the genome of which is being completed) and in citrus will reveal the HLB-specific markers in citrus.

Expected Deliverables: Validated markers that will be used for the pre-symptomatic diagnosis of HLB using various advanced platforms that have already been developed or being developed under the sponsorship of USDA-APHIS and citrus industries. In addition, genetic factors that confer HLB tolerance and/or resistance will be engineered into citrus for protection against HLB.

Contributors: Gupta, Davis, Stover, McCollum, Lin, Gmitter, Bowman, Grosser, Jin.

Use of psyllid genome information for HLB bio-control

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Outcome Group 3

Introduction: Psyllids (Order Hemiptera) are phloem-feeding insects, many of which are important plant pests. However two, *Diaphorina citri* (the Asian citrus psyllid) and *Bactericera cockerelli* (the potato psyllid) are of relevance here because they are the vectors of phloem-limited bacteria which are associated with important plant diseases. *D. citri* transmits *Ca. Liberibacter asiaticus* (and some related candidate species) which is associated with citrus greening, and *B. cockerelli* transmits *Ca. Liberibacter solanacearum* which is associated with zebra chip of potatoes and with tomato psyllid yellows. These diseases are very economically important and information is greatly needed in order to develop effective, environmentally sound approaches for controlling them. Contemporary sequence data and analyses can contribute to these goals.

The potential of genome sequence data for psyllid control: These are the days of rapid, efficient, high throughput next Generation sequencing. This is in part because the genome of a specific organism (e.g. *D. citri*) is a master blueprint that details the specific biochemical pathways, biological and metabolic processes that offer invaluable information on the identity and biology of a particular organism. But more importantly, technological and scientific advances have made it such that gaining the genome (and transcriptomic as well as small RNA profiling) information of *B. cockerelli* and *D. citri* is a very do-able objective, that can be accomplished relatively quickly and cost effectively. The resulting sequence data combined with contemporary bioinformatics analyses offers great potential for fundamental biology, but also for developing new strategies for psyllid (and associated plant disease) control. Because the genome sequence is specific to a given organism, these data can be used to ensure efficacy and target specificity (e.g. targeting only *D. citri*) for any sequence-based psyllid control approaches, things that will be critical for any regulatory approval. Furthermore, transcriptome data for the various *D. citri* instars and even different organs will provide important information including transcript abundance in different tissues (e.g. the gut) and or life stages (e.g. nymphs vs. adults). For example, if RNA interference (RNAi)-based approaches are to utilize oral delivery/acquisition of the effector RNAs, it is likely that psyllid gut-specific RNA targets will be more susceptible than will targets which are more general or more abundant in tissues distal from the gut. Similarly, if psyllid nymphs prove to be more susceptible to RNAi than are adults, targeting RNAs identified to be important for nymphs could prove to be more efficacious. Small RNA sequence information relative to the transcript locations and abundance also will be critical for identification and design of highly efficient sequence-based targets, and will help to ensure our ability to design correct RNAi effectors. Additional opportunities include designing strategies to block *D.*

citri receptor uptake and/or transmission of *Ca. L. asiaticus*, or interfering with *D. citri*'s ability to locate and establish phloem connections in citrus.

Transcriptome sequencing from two different psyllids (*D. citri* and *B. cockerelli*) also provides us an opportunity to identify the SNP (Single Nucleotide Polymorphism) variations between the two organisms that can invariably contribute to the design of control strategies. SNP variations also would help us understand the variations in the associated pathogen complex that are either evolving or might be able to provide the clues on pathogen virulence associations. Comparative genomes also would aid us in understanding deletions or rearrangements of large segments of the genome which indicates the evolutionary significance of the populations and their ability to transmit the corresponding *Ca. Liberibacter* species to plants.

Sequencing the psyllid transcriptome and small RNA-ome offers additional benefits. For example, the resulting sequence data will not only include sequences of the psyllid, but also of any associated microbes including fungi, prokaryotes and even viruses. If microbial transgenesis (of either endosymbionts or even *Ca. L. asiaticus*) is to be considered as a desired outcome, the microbial sequence data will be essential to design effective transgenesis approaches. A new opportunity also includes identifying previously unknown microbes, in particular psyllid-infecting viruses. Viruses are the most abundant microbes on the planet and have been reported from all organisms examined so far, but only one virus is currently known from *D. citri*, there have to be more. If we can identify additional *D. citri*-infecting viruses via next generation sequencing, we might be able to engineer a virus (or viruses) to express toxic RNAs and/or peptides within *D. citri*. This offers potential particularly in RNAi opportunities. As recombinant viruses replicate and move throughout the psyllid body they would induce RNAi effects in all infected tissues, not just the gut, thereby possibly resulting in death of *D. citri*. Alternatively, it is conceivable to engineer a virus to express peptides which could be toxic to either *D. citri* or *Ca. L. asiaticus*. The latter might negatively impact the ability of *D. citri* to transmit *Ca. L. asiaticus* to plants. Prokaryotic endosymbionts might also be engineered for similar goals.

What do we need? We need complete comparative sequence data for *D. citri* and *B. cockerelli* and associated microbes (*Ca. L. asiaticus*, endosymbionts, viruses) as soon as possible. This includes transcriptome and small RNA sequences from diverse *D. citri* populations. This requires collaborations among scientists, but also the necessary sufficient and consistent funding to support the specimen collection, sequencing and bioinformatic analyses, and the biological application approaches. This is not a one year effort.

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Functional and comparative genomics of Liberibacters

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Outcome Group 3

Bacterial phages are horizontal gene transfer vehicles that can create new and emerging plant diseases by carrying specific genes that allow a recipient bacterium to acquire traits needed to colonize a new ecological niche, including new hosts. Two prophages were found in Las, Lam and Lso, and at least in the case of Las and Lam, at least one appears to replicate as an excision plasmid prophage. A replicating plasmid form was found in both Las and Lam; in Las, the replicating form was found in infected periwinkle, citrus and psyllids. Although there are relatively few genes present in Las, Lso and Lam that would be categorized as defensive, the replicating prophage carries putative lysogenic conversion genes---specifically, (defensive) peroxidases. The generation of reactive oxygen species (ROS), including superoxide radicals, hydrogen peroxide, and hydroxyl radicals is among the first lines of defense used by insects and plants against invading microbes. ROS production is significantly upregulated in Wolbachia-infected mosquito cells compared with non-infected cells. Circular forms of both phages were found in Lam, indicating that as with Las, both phages have the potential to become lytic in plant infections. Heat treatment was recently shown to “cure” Las from infected citrus, and phage lytic cycle activation was implicated (refer Y.P Duan, ARS). Hypervariable regions were found in the phage of different Las strains from Florida and from global samples, and at a minimum these regions may be used for strain identification purposes (refer Y.P. Duan, ARS). Although lytic cycle genes are not expressed in prophages, non-lysis, non-structural and non-virion assembly genes typically are expressed, and plasmid replication allows gene amplification.

Liberibacter detection and HLB diagnosis

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Outcome Group 1

Reliable *Liberibacter* detection and Huanglongbing (HLB) diagnosis are critical for effectively managing the disease to keep orchards with the Asian citrus psyllid HLB productive. Typically a diagnosis that the disease HLB is affecting a citrus tree is dependent upon evaluation of foliar symptoms, with confirmation that the appropriate vector is present and that DNA of the HLB-associated *Liberibacter* is detected in nucleic acid extracts from symptomatic tissue. Real-time PCR (qPCR) assays for the detection and discrimination of the three species of *Liberibacter* associated with HLB were developed and implemented quickly after the confirmation of the disease in Brazil and Florida in 2004 and 2005 respectively. Alternatives to direct visual assessment for disease evaluation and to qPCR for pathogen detection have been proposed and developed to varying extents. Non-destructive procedures for evaluating the infection status of a given tree that are under evaluation and development include 1) detection of differences in the profile of volatile organic compounds released from infected and non-infected trees as well as 2) hyperspectral imaging and 3) detection of infection-associated small RNAs and metabolites that may be markers of infection prior to detection of the associated pathogen and the development of symptoms. Current methods of pathogen detection are destructive by necessity and the most common method of establishing the presence of *Liberibacter* by the detection of pathogen DNA is qPCR. Additional nucleic acid-based procedures which have been developed and tested include 1) Multiplex Oligonucleotide Ligation-PCR (MOL-PCR) which is based on a standard DNA amplification methodology and 2) and a process of amplification of the target sequence based on the use of reverse transcriptase and T7 RNA polymerase. Antibody based methods that have been developed and are under evaluation make use of anti-HLB antibodies in an easy to use cassette format. Some of these innovations will be useful for both field and research applications whereas others may be more limited in application and use. Future research leading to improved *Liberibacter* detection and HLB assessment should focus on pre-symptomatic or clinically symptomless HLB-affected trees.

Using chemical ecology tools to prevent or slow the spread of ACP and HLB

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Outcome Group 2

This presentation will discuss state of the art chemical ecology, including gaps in knowledge, as it is applied to the problem of the Asian citrus psyllid. The speaker will describe the current efforts in the development of more efficient chemical ecology tools to manage this vector and their implications. Possible behavioral mechanisms at play with ACP, possible candidate semiochemical formulations and associated management techniques to better monitor, prevent or slow the spread of ACP and HLB will also be discussed.

HLB therapeutics

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Outcome Group 1

Huanglongbing (HLB) disease is the most severe economic strain/concern for citrus producers in the U.S., and as a result, a myriad of solutions are being sought to reduce its economic impact. Although development of trees that are resistant to the disease and/or the Asian citrus psyllid vector is seen as a needed long-term solution, therapeutic treatments to citrus are being sought that either kill, or reduce the fitness of, the bacterium ("*Candidatus Liberibacter*" species) associated with the disease; block psyllid acquisition/transmission of the pathogen; and/or, improve the trees ability to tolerate the disease. This presentation will be limited to those strategies that are being evaluated for their potential to be used as therapeutic treatments of trees without genetic engineering or further plant breeding. Topics to be presented include: 1) Research on identification of antimicrobial compounds that also includes screening and identification of antimicrobial compounds effective against the phloem-limited "*Candidatus Liberibacter asiaticus*" bacterium and development of application strategies to improve uptake of antimicrobials into the citrus phloem; 2) Results from two Florida citrus industry sponsored screening contests that have led to the identification of new compounds/molecules that could be used either to control the bacterium or for psyllid control (RNA inhibition inducing dsRNAs) in therapeutic strategies; 3) Research on optimizing the delivery of therapeutic agents to the phloem; and 4) An overview of the status of thermal therapeutics and an array of foliar nutrition treatments that are being evaluated in Florida.

Outcome driven discussion: Model systems for studying HLB

Ed Stover with input from a cast of thousands

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Outcome Group 3

After intentional inoculation of citrus with *C. Liberibacter asiaticus* (CLas), through grafting with infected budwood or exposure to infected psyllids, it takes 6 weeks to as long as 6 months to have readily detectable pathogen and 10-24 months for good symptom development. Even then, a typical response would be that 60-80% of the inoculated plants show infection. Since the citrus / HLB pathosystem is challenging and slow, it would be extremely advantageous to have a faster more efficient model system, and a number of approaches to model systems have been explored. The discovery of *C. Liberibacter solanacearum* (aka *L. psyllaureus*; CLsol) as a pathogen in solanaceous plants has provided the most extensively studied model system for HLB. It provides almost an exact parallel to HLB in citrus, with plant hosts displaying phloem infection following inoculation with *Liberibacter* through a psyllid vector. It has the additional advantages that it is an important disease in its own right, it can be studied in areas where concerns of inadvertent spread prevent direct study of HLB, and host plants are more quickly produced following transformation with strong genomic resources for some.

Manjunath et al. report that symptoms appear in tomato 2-3 weeks after infection. They further report identification of resistant and tolerant varieties, that plant infection is uniformly systemic, and that psyllids are ~100% infected. Mirkov has studied tobacco/CLsol as a model system for evaluating transgenics and demonstrated resistant transgenics within 2 weeks and discrimination in level of transgenic resistance at 4 weeks. Belknap and Munyaneza used potato/CLsol as a model HLB system for evaluating transgenics and found no effect. They suspect they overwhelmed the potatoes with excessive inoculum and are repeating their experiments at lower CLsol load in the psyllid vectors. Roose and Patne explored *Arabidopsis* as a host for CLsol, to harness the enormous information known in this model plant. Screening of chemicals that induce resistance/tolerance was impeded by inability to infect cultured plants with psyllids and high cost of chemical for soil-grown plants. They have transitioned to a tomato system. Lin and Mwugo studied the proteomic and nutritional response of potatoes to CLsol infection: 50+ proteins were differentially expressed with up-regulation of proteins involved in pathogen-response/defense, molecular chaperones, and energy production/general metabolism. Down-regulation of protease inhibitors in potato tubers and photosynthesis-related proteins in potato leaves, correlated with a reduction in Mg, Ca, and Zn concentrations. Wuriyangan et al. studied RNA silencing in the CLsol psyllid vector. They targeted ubiquitously expressed and gut-abundant mRNAs with double-stranded RNAs and siRNAs and were able to induce mortality in psyllids, with knockdown of target mRNAs, and accumulation of BC-Actin mRNA-

specific siRNAs. Zhang et al. studied a periwinkle model system vs. citrus itself as materials for screening chemicals with therapeutic suppression of CLas. While the periwinkle system required a shorter incubation time and permitted rapid propagation, they conclude that the citrus system is superior as a direct assay in the system of interest.

The Dawson lab has worked extensively with CTV expression vectors, both as therapeutic delivery agents and tools to screen potential transgenes. The Shatters lab has worked with screening therapeutic materials against CLas or specific step in the pathosystem through artificial feeding to ACP, which would ultimately be implemented as transgenic product or applied exogenously in-planta. Early results show that feeding of ACP on RNAi directed at blocking CLas uptake shows significant differences from controls, but appears to underestimate effectiveness when delivered in-planta. The Stover lab has worked with evaluating antimicrobial peptides (AMPs) using in-vitro cultures of *Agrobacterium* and *Sinorhizobium* which are related to *Liberibacter* but easy to culture. Results have been used by Jaynes to refine design of AMPs that show promise in CTV expression vectors and may have value expressed transgenically. Ammar, Walter and Hall used an excised-leaf assay to speed up assessment of ACP transfer of CLas into leaves from 3-12 months to 2-3 weeks. Leaf stems were inserted into small tubes containing water and leaves were maintained in ventilated tubes with known infected ACP. After 1-2 weeks of exposure to 5-10 ACP an average of ~40% of leaves were CLas+. This procedure is relevant to just a few steps in the pathosystem, but could be useful in evaluating whether genotypes or treatments prevent feeding or transfer of CLas from ACP. A tremendously powerful model system would be incorporation of therapeutic agents into reliable cultures of *Liberibacter*. This remains intractable to date, but merits further effort.

Progress on the cultivation of *Liberibacter* in vitro

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Outcome Group 1

The current CRDF-funded efforts to culture *Ca. Liberibacter asiaticus* (CLas) will be summarized. Four labs have independently shown that CLas can be cultured for a few days prior to culture death. CLas cells change morphology during this process. The phages present in the CLas genome may be responsible for the early culture death. Evidence for this will be discussed as well as ways to avoid phage inhibition of culture growth. Another new development is the use of microfluidics to aid in culturing CLas. Meanwhile, the genome of a very close cultured relative of CLas has been sequenced. This strain has been characterized phylogenetically, physiologically, and morphologically. Its close relationship to CLas and related pathogens suggests that it is in the same genus. Hence, the name *Liberibacter crescens* is proposed for this organism. This cultured strain may serve as a good surrogate for high throughput, in vitro screening of antibiotics. Effective antibiotics against *L. crescens* can then be screened in planta for effectiveness against CLas. So while progress is being made toward the culturing of CLas, a non-pathogenic, close cultured relative is available for a wide variety of comparative analyses.

Potential application of area-wide management programs for controlling the Asian Citrus Psyllid (ACP) and Huanglongbing (HLB)

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Outcome Group 2

Area-wide pest management programs (AWPM) have several components in common to be successful. The most important component of a successful AWPM program is active grower participation with full engagement by the public sector to deliver mission-linked research, extension, education and training. The advantages of an AWPM must be demonstrated and scaled up for the end users impacted by pests. The expected outcome is adoption of IPM strategies into sustainable systems, increased profits for industry, health and environmental benefits, and reduced reliance on chemical pesticides. Insect and weed pests that have been effectively managed through the USDA-ARS AWPM initiative include: the codling moth on apples and pears, corn rootworms in the Midwest, leafy spurge, Tephritid fruit flies in Hawaiian Islands, fire ants in the southern U.S., *Melaleuca* in the Florida everglades, Asian tiger mosquito in NJ, etc. However, not one of these programs involved an insect vector that transmits a pathogen to a perennial crop, as the case for ACP/HLB. Information and updates from current programs will be presented on the Citrus Health Management Areas (CHMAs) in Florida, and the current strategies for ACP suppression and HLB management in Texas and California. Examples from the Asian tiger mosquito programs will be presented to illustrate the challenges of urban environments.

Poster Abstracts

Effect of temperature on lighted sticky traps (TransTrap®) used to detect Asian citrus psyllids in shipping containers

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Outcome Group 2

The Asian citrus psyllid (ACP) is an insect native to tropical and subtropical regions in Asia, which has spread into most citrus producing regions around the world. The main concern with ACP is the spread of Huanglongbing (HLB), a disease vectored by the psyllid. The volume of citrus shipments from Mexico into the U.S. prompted questions about the risk of moving ACP and HLB on this pathway. Our goal was to determine temperatures at which ACP would be active and thus fly to lighted sticky traps inside a transport trailer.

Our results found that temperature significantly affected the number of adults captured. No psyllids were recovered on the traps at 12 °C (53 °F), which is the average temperature of citrus shipments crossing the border. This is compare with an average capture of 43, 47, and 42% of the released adults at 24, 28, and 32 °C (75, 82, and 90 °F), respectively. Only 1%, 2% and 18% were captured at 18, 20 and 22 °C (64, 68, and 72 °F), respectively. Given that the average temperature of limes arriving at the border is 12 °C, it is unlikely that ACP adults would fly to a lighted sticky trap placed inside a refrigerated trailer. In addition, the majority of the packing houses in Mexico place the fruit into coolers prior to loading. We believe a lighted sticky trap would not be effective in detecting ACP within refrigerated citrus shipments for the period of time from loading at the packing house to trap recovery at the border crossing.

Phylogeographic analysis of Asian citrus psyllid, a vector of citrus greening disease

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Outcome Group 2

Citrus greening, or Huanglongbing, is one of the most serious diseases of citrus. Caused by bacteria of the genus *Candidatus Liberibacter* spp., the disease reduces production, destroys economic value of fruit and kills trees putting at risk the U.S. citrus industry valued at \$1.3 billion annually. The bacteria are vectored through feeding activity of the Asian Citrus Psyllid, *Diaphorina citri*. This widespread psyllid was first found in Florida in 1998 and followed by Puerto Rico and the U.S. citrus producing states. Soon after, citrus greening was found in Florida in 2005 and subsequently most of the US citrus producing states, excluding Alabama, Arizona, California and Texas. Given its rapid spread and devastating impact, it is surprising that while research has explored the vector's host plant range and interactions with host, parasitoids and pathogen, little effort has gone into exploring psyllid genetic diversity and pathways of movement. Here we present the initial stages of a phylogeographic analysis of *D. citri* in an attempt to gain understanding into its geographic origins and pathways of movement.

Transcriptomic differences between HLB-affected citrus varieties with different sensitivity

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Outcome Group 3

Citrus Huanglongbing (HLB, presumably caused by *Candidatus Liberibacter asiaticus*; CLas) is considered the most serious problem for citrus production. There is no known resistant germplasm although some citrus types appear to be more tolerant and/or insensitive to the disease, exhibiting slow decline and a capacity for rejuvenation. To understand and compare the transcriptional differences of the underlying mechanisms, microarray analysis of tolerant rough lemon (*Citrus jambhiri*) and susceptible sweet orange (*C. sinensis*) in response to CLas infection was conducted. More genes were differentially expressed in HLB-affected rough lemon than sweet orange at the early stage, but substantially fewer were at later time points, possibly a critical factor underlying differences in sensitivity to CLas. Pathway analysis revealed that stress responses were distinctively modulated in rough lemon compared with sweet orange. The different numbers and fold changes of differentially expressed genes between the two varieties at different time points may be contributing to the differences in HLB symptom development as well as the rate of decline. The results potentially lead to identification of key genes and the genetic mechanism that enables rough lemon to restrain disease development and maintain (or recover) phloem transport activity. These potential candidate genes may be used for improving citrus tolerance (or even resistance) to HLB by genetic engineering, and provide clues on early signaling events that could be developed into diagnostic tools.

Characterization of the closest cultured relative *Ca. Liberibacter asiaticus*: A possible surrogate for in-vitro studies

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Outcome Group 1

The gram negative, rod-shaped bacterial isolate, BT-1 was recovered from Papaya exhibiting Papaya Bunchy Top-like symptoms. Sequencing of the 16SrRNA gene showed this isolate was not the causal agent of the disease. However, the BT-1 16S rRNA sequence showed that this strain is more closely related to *Ca. Liberibacter* than any other strain in culture sharing 96% homology with *Ca. Liberibacter solanacearum* and 95% homology with *Ca. Liberibacter asiaticus* which cause Zebra Chip (ZC) and HLB diseases, respectively.

Whole genome sequencing of BT-1 revealed further similarities between it and *Ca. L. asiaticus* as well as *Ca. L. solanacearum*. Sequence-based comparisons show that 75.5% and 73.1% of the predicted genes in *Ca. L. asiaticus* and *Ca. L. solanacearum* respectively are also found in the BT-1 genome. Further phylogenetic analyses suggest that BT-1 is a member of the same genus as the HLB and ZC pathogens. Hence, the name *Liberibacter crescens* is proposed for the BT-1 isolate. We also propose that the high degree of genetic and metabolic similarity between *L. crescens* and CLas makes BT-1 a potential culturable surrogate for the in vitro study of *Liberibacter* pathogens. In particular, BT-1 may be very useful for rapid antibiotic sensitivity profiling that would suggest useful antibiotics for the treatment of the disease in the field. There is no evidence that BT-1 is a citrus pathogen.

Evaluation of thermotherapy against Huanglongbing (citrus greening) under laboratory conditions

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Outcome Group 1

Huanglongbing (HLB, Yellow shoot disease) is the most destructive disease of citrus. The disease is associated with “*Candidatus Liberibacter asiaticus*”. Few management options are available, besides preventive measures such as the removal of affected plants, planting disease-free stock and maintaining vector-free production in quarantine areas. In this study, we assessed the efficacy of thermotherapy against the disease under controllable laboratory conditions. A total of sixty, 2-year old graft-infected Lu tangerines, Citrus reticulate Blanco, seedlings were used for the study. The plants were randomly divided into 3 treatment groups (45, 48°C, and untreated), with 5 plants/rep, 4 reps/trt. The treated plants were placed in temperature chambers for a 4-h treatment session, repeated weekly 3 times. Disease remission was observed beginning 8 weeks post-treatment. Real-time PCR assays revealed that pathogen (“*Ca. L. asiaticus*” concentration of all the HLB-affected seedlings were significant reduce except 8 plants under 45 and 48oC treatments at 4 weeks after treatment. In contrast, pathogen concentration in the untreated control plants exhibited a significant increase, with the highest increase of about 30-fold compared to the initial pathogen concentration (pre-treatment). Except for 7 plants (7 out of 40 total plants), pathogen concentration in the new flushes of the treated plants decreased 90% at ca. 8 weeks after treatment, compared to the initial pathogen concentration. Nested and real-time qPCRs were used for confirmation of HLB infection in the seedlings, and for pathogen titer assessment. Although the result is considered preliminary, it provides a foundation for further work in developing the technique for HLB management. Complementary work will include exploration of additional exposure time and temperature combinations as well as treatments using commercial field settings.

Evaluating the biological control of ACP in the Rio Grande Valley of Texas

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Outcome Group 2

Tamarixia radiata, a species specific ectoparasitoid of the Asian citrus psyllid (ACP), *Diaphorina citri*, was imported from Pakistan and permitted by the PPQ Permitting Unit for field release in Texas. Since September 2011, over 100,000 parasitoids have been released in the Lower Rio Grande Valley. Of these, over 35,000 have been released within a 5-mile radius where citrus greening disease was detected in January 2012. Both open and closed releases are conducted and used to assess establishment and efficacy. Closed releases made in fine-mesh sleeve cages indicate parasitism levels at 10.4%. When compared to the controls, host mortality is reported at 64.9% in cages with parasitoids present versus 4.4% in cages with parasitoids absent. Further investigations into the host mortality of ACP nymphs have been explored by conducting visual observations on the behavior of female parasitoids ($n = 30$) for one-hour periods in arenas with suitable hosts. Data indicates that females will mount 3.1 ± 0.5 nymphs per hour. The parasitoid will either oviposit the nymph on the ventral side (36.5% of the time) or probe the nymph on the dorsal side (63.5% of the time). After probing, the parasitoid will either walk away (87.9% of the time) or host feed (12.1% of the time). Host feeding was documented at 0.43 ± 0.1 nymphs per hour. All nymphs that were host-fed were found to be eventually dead. Host mortality (64.9%) and parasitism rates (10.4%) combined can reduce ACP populations by 75.3%. Studies are still ongoing to help reduce both ACP populations and the incidence of citrus greening disease.

Functional and comparative genomics of Liberibacters

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Outcome Group 3

There are two complete circular genomic DNA sequences of Liberibacters deposited in GenBank. The first is *Ca. L. asiaticus* (Las), and the second *Ca. L. solanacearum* (Lso). Recently the complete genomic sequence of a third, '*Ca. L. americanus*' (Lam) strain "São Paulo" was completed (refer Wulff et al poster). At least 3 other genomic projects are in progress: a second Las (China), a second Lam, and *Ca. L. africanus* (Laf; refer H. Lin, ARS). Las is widely distributed and heat tolerant, the Laf is less widely distributed and produces no symptoms above 30 °C., and Lam is now recognized as also being heat sensitive. A unique cold shock protein is present in Lam that is not in Lso or Las. The overall gene organization and structure of the Lam genome is more similar to Lso than to Las, but Lso and Las are more closely related to each other than to Lam, despite the difference in host range. Surprisingly, comparative analyses revealed no obvious virulence gene candidates that might be attributed to host range on citrus (ie., common to Las and Lam but missing in Lso). There is strong evidence of genome degeneration in Las, Lam and Lso, with many apparent pseudogenes or truncated genes found among the unique genes of all 3 species. Comparisons with other alpha proteobacteria reveal some proteins unique to Las are a set of predicted genes for the biosynthesis of LPS and a set of seven genes for the synthesis of cholesterol (refer J. Hartung, ARS). Interestingly, most of the predicted genes required for LPS biosynthesis in Las appear to be missing in Lam.

Attraction of Asian citrus psyllid to traps in urban environments

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Outcome Group 2

Current presence/absence detection method for Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), in urban areas relies on the use of yellow sticky traps without additional chemical lures. This trapping method is used because in absence of data for an urban setting, and is acceptable for use in commercial citrus production. Detection of adult Asian citrus psyllid in urban areas is essential to reduce the spread of the psyllid from urban areas to surrounding commercial citrus production. Therefore, we investigated the relative trapping efficiency of four traps (four different colors) currently used for detection of Asian citrus psyllid adults. We found no statistically significant differences among the trap catches of the various colored traps. So, when we investigated the attractions of traps with the addition of two chemical lures that mimic plant volatiles from Eureka lemon and Mexican lime, we used a yellow sticky trap. The studies were conducted in urban areas in Los Angeles, California in the spring and summer of 2011. The results of the studies are discussed in terms of use in a presence/absence detection method.

Evidence- and risk-based surveys for ACP/HLB: Adaptive modeling and impact analysis

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Outcome Group 1

Evidence-based risk assessment is a key part of any effort to predict where ACP and HLB are likely to occur and to plan sampling protocols for detection and surveillance. The USDA-ARS sub-tropical plant pathology laboratory has constructed a GIS-based risk model for ACP and HLB that combines several categories of evidence and uses both mechanistic and empirical elements to generate risk indices for individual STR (section-township-range) 1mi²-grid cells. The risk model is adaptive and essentially Bayesian in its design in that it can be initiated from best guesses for risk parameters to generate initial estimates of risk in the absence of empirical data. However, if data are available from the outset (from previous sampling for example), or as they accumulate from sampling, the model algorithm combines the information from the ground-truth samples with the risk parameters to adapt risk estimates on the basis of the data. In addition to modeling ACP and HLB risk, the system can be used to calculate human resources required to provide different levels of sampling effort and to target control/mitigation responses. We are further extending the model to include estimates of economic impact resulting from ACP or HLB presence and for different control methods, to further augment its value as a strategic tool for disease management.

Asian citrus psyllid management strategies for California

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Outcome Group 2

Asian citrus psyllid (ACP) was first detected in southern California in September 2008 in the urban landscape. Since that time, the California Department of Food and Agriculture has conducted an eradication program utilizing systemic imidacloprid and foliar cyfluthrin wherever psyllids are detected. This program was halted in Los Angeles County in 2011 because of the size of the psyllid infestation. Psyllids have continued to spread to the east and the south into San Bernardino, Riverside, and Orange Counties. It is hoped that biological control will help reduce ACP in urban areas of LA. In areas such as San Diego and Imperial where urban treatments have been continuous, detections of ACP in commercial citrus are rare. In contrast, in San Bernardino County, where psyllids may spread from Los Angeles and where the treatments are not well-coordinated in an area-wide fashion, psyllids are becoming established in both urban and commercial citrus. The University of California in collaboration with the California Citrus Pest and Disease Prevention Committee have developed strategies for managing ACP once it establishes in commercial citrus. In the initial phase of invasion, when ACP densities are low, aggressive applications of two broad spectrum insecticides are recommended. In areas where the psyllid has become established, area-wide treatment programs utilizing 3-5 insecticides/year are necessary. ACP treatments are directed at periods of flush and also at the late fall and early spring overwintering populations. Insecticide choices are based on considerations of efficacy, control of other pests, costs, preservation of natural enemies and resistance management. A sampling plan and treatment program for organic citrus is under development.

A preliminary study on using plastic sheeting for reducing population of ‘*Candidatus Liberibacter asiaticus*’ associated with Huanglongbing (citrus greening) in Guangdong Province, China

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Outcome Group 1

Asian type Huanglongbing (HLB, also citrus greening), which is associated with bacterium *Candidatus Liberibacter asiaticus* (Las), is considered as heat-tolerant, so is the insect vector, Asian Citrus Psyllid (ACP), *Diaphorina citri* Kuwayama. However, recent studies suggest that high summer temperature appears to be a significant factor restricting HLB occurrence and development. We therefore proposed a novel heat-based approach for managing HLB-affected groves: thermal therapies delivered via temporary plastic sheeting powered by natural sunlight. A total of 24 sweet orange trees with HLB symptom was selected, and divided into six blocks, with 4 trees per block. Three blocks (replicates) were used for the plastic sheeting treatment, and another three blocks were used for untreated control. A randomized complete block design was used for assigning each of the blocks. The real-time PCR was used for assessing Las populations. The results indicate that plastic sheeting treatment reduced Las population significantly. Average Las copy number was reduced by 85.0, 83.3, and 85.2%, respectively, in three consecutive months (Oct, Nov, Dec) post-treatment. In comparison, Average Las copy number in the untreated group reduced by 19.0, -12.7, and 25.0%, during the time. Further field and lab studies are being conducted to evaluate the efficacy of plastic sheeting for control HLB.

High-throughput screening of transgenic and conventional citrus for HLB and ACP resistance

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Outcome Group 3

USDA-ARS in Fort Pierce, Florida has produced thousands of scion or rootstock plants transformed to express peptides that might mitigate Huanglongbing (HLB); additional plants are being continually produced. The more rapidly this germplasm can be evaluated, the sooner we will be able to identify transgenic strategies for controlling HLB. Therefore, a high-throughput facility to evaluate transgenic citrus for HLB- and ACP resistance has been established by USDA-ARS-USHRL. Stage I - Individual plants of each transformed genotype are replicated and caged with infected psyllids for one week (20 or more per plant depending on the percentage that test positive in an individual colony). Stage II – Cages are removed from the plants and the plants are housed for six months in a greenhouse with an open infestation of infected psyllids. Stage III - Plants are then moved into a psyllid-free greenhouse and evaluated for growth, the presence and titer of the HLB bacterium '*Candidatus Liberibacter asiaticus*', HLB-symptoms, and where appropriate monitored for ACP colonization. Thanks are extended to CRDF for partially funding this project.

Within plant distribution of *Candidatus Liberibacter asiaticus* in HLB-infected citrus trees in Texas

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Outcome Group 1

The Asian citrus psyllid, *Diaphorina citri* Kuwayama, one of the vectors for citrus greening disease or Huanglongbing (HLB) has been present in Texas for over a decade, but the detection of the disease is recent. HLB has been confirmed in only two adjacent commercial citrus groves of grapefruit and sweet orange. A study was conducted to compare the population of *Candidatus Liberibacter asiaticus* (CLas) cells in different plant parts including pedicil, columella, leaves, seeds, young shoots, flower buds, flowers, and bark of known infected grapefruit and sweet orange trees. The bacterial population was derived using a previously described grand universal regression equation $Y=13.82-0.2866X$, where Y is the log of the target copy number and X is the Ct (threshold cycle) of the assay. Overall, significantly higher CLas cells were recorded in grapefruit than sweet orange. The bacterial concentration also varied with the plant part; pedicil, columella, midrib having significantly higher titer of CLas compared to other plant parts. This is in agreement with previous studies conducted on Florida samples, but a new finding was that lowest bacterial titer was consistently recorded in young shoots and leaf blade, especially leaf margins.

A model for the spread of HLB attributed to the vector, *Diaphorina citri*

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Outcome Group 2

A model of the transmission of HLB between the psyllids and trees in a citrus grove is developed to attempt to understand the spread of HLB in Florida. We simulate the spread of HLB in a single grove and focus on the impact of the following factors: long distance migration and local movement of the psyllid vector, vertical and sexual transmission of Las among psyllids, and the difference between the latent period (time between initial infection and the point at which psyllids can acquire the pathogens) and incubation period (time between initial infection and the point at which symptoms appear) of HLB in the trees. We are particularly concerned with whether the vertical and sexual transmission could be a factor for the rapid rate at which Florida saw the disease spread in its early stages. We also show that if the difference between the latent and incubation period of the disease in the tree is large, then the removal of trees in a grove may not be a reasonable management strategy. One should then consider tradeoffs among combinations of interventions that involve nutrient management of trees, psyllid control, and removal of trees.

Detection of *Liberibacter asiaticus* in a single infected Asian citrus psyllid adult or nymph: Impact of dilution with clean Asian citrus psyllids (*Diaphorina citri*) during extraction

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Outcome Group 2

The presence of Huanglongbing (HLB), a devastating citrus disease, has been confirmed in California. Protecting the state's citrus industry through detection and removal of infected trees is essential in curtailing its spread. Because 'Candidatus Liberibacter asiaticus' (CLAs), the putative causal agent of HLB, reproduces in its vector, the Asian citrus psyllid (ACP), *Diaphorina citri*, Kuwayama, accurate testing of the insect is vital. The method of testing is quantitative polymerase chain reaction (QPCR) targeting a region of the CLAs 16S ribosomal gene. Because insect secondary metabolites interfere with downstream applications, there is concern about the number of insects pooled in DNA extractions without compromising CLAs detection. We are determining the pooling limit through experiments using CLAs infected ACP from a colony at USDA-ARS in Fort Pierce, Florida and a clean UC Riverside, California quarantine colony. In addition, Candidatus Liberibacter solanacearum (CLsol) infected and clean potato psyllids, *Bactericera cockerelli*, from colonies at the USDA-ARS Repository in Riverside will be studied as well. Individual insect DNA extracts from the infected colonies are evaluated by QPCR to identify positives and pooled. DNA equivalent to a positive psyllid is added to either 4 or 24 intact clean psyllids and extracted and the presence of *Liberibacter* determined by QPCR. Potato psyllids generally acquire CLsol at rates of 100%. Therefore, single intact potato psyllids from the CLsol positive colony have been extracted with 4 or 24 clean potato psyllids and CLsol determined. Depending on the percent of infected ACP, this experiment will be repeated with ACP.

Multiple genome sequencings of HLB-associated ‘*Candidatus Liberibacter*’ species

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Outcome Group 3

The objective of this project is to obtain the complete genome sequence of multiple “*Candidatus Liberibacter*” species that are associated citrus HLB. There are three species of ‘*Ca. Liberibacter*’, each named based on its presumptive origin. ‘*Ca. Liberibacter asiaticus*’ (Las) is believed to originate from Asia, ‘*Ca. Liberibacter americanus*’ (Lam) from the Americas, and ‘*Ca. Liberibacter africanus*’ (Laf) from South Africa. Currently Las is found in Asia and the Americas. In the United States, HLB was first discovered in Florida in 2005. Since the first report, the disease has been spread to other citrus growing states including Texas and California. Successful management of HLB requires development of molecular diagnostic tools for accurate and early detection of HLB-affected trees and a complete understanding of factors affecting pathogenicity. Although the genome sequence of a Florida Las strain is available, genome sequences of the other two *Liberibacter* species are not available. Using Illumina sequencing technology and genomic DNA enrichment technique, we have successfully obtained complete genome sequences of a Las strain from China, a Laf strain from South Africa and a Lam strain from Brazil. Genome sequences were *de novo* assembled by Velvet and SOAP software. All *Liberibacter* sequence contigs were *in silico* identified and reconfirmed by PCR. We are now reconstructing a complete *Liberibacter* chromosome for each species via PCR-based gap closing methods. The complete genome sequences and comparative analyses of all three *Liberibacter* species will provide new insights into the genome evolution of *Liberibacter* species, enable development of improved diagnostic tools, and facilitate identifying critical molecular mechanisms responsible for the disease.

California's response to the first detection of HLB

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Outcome Group 2

Since the first detection of the Asian Citrus Psyllid in California in August 2008 the California Department of Food and Agriculture has implemented, in addition to the long-standing ACP trapping program, visual surveys, psyllid collections and chemical treatments within a 400m radius of all psyllid finds. Despite the surveys and suppression efforts within two years the psyllid had spread throughout five Southern California counties, principally in Los Angeles County, but not to the Central Valley. In March 2012 a psyllid collected from a grafted pummelo (of unknown origin) during a visual survey in L.A. County tested positive for HLB, the first such find in California. Plant material collected from the tree confirmed that the tree was positive for HLB. Eradication of the tree ensued paralleled by the CDFA developed HLB-positive protocol previously established for all citrus and citrus relatives, ACP nymphs and adults within an 800m radius were collected and tested. With the positive HLB find the Citrus Pest Disease Prevention committee, working with CDFA, provided the funding for HLB testing in three zones: 1) test all host plants and ACP (adults and nymphs) in the first 400m around the find 6 times/year, 2) test host plants and ACP in the next 400m three times/year, and 3) survey 50% of host plant and ACP in the next 400m twice/year. To date no other HLB-positive psyllids or trees have been detected.

Citrus gene regulatory networks to clarify Huanglongbing disease mechanisms

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Outcome Group 3

The power of next-generation sequencing technologies was exploited to gain deeper insight into how fruit and leaf gene networks respond to infection by *Candidatus Liberibacter asiaticus*, agent of Huanglongbing disease (HLB). Four categories of orchard trees were studied: symptomatic, asymptomatic, apparently healthy, and healthy. PCA analysis determined that 1063 of the 77,116 predicted genes distinguish the fruit and leaf HLB-regulated transcriptome. A predicted protein-protein network identified HLB-regulated genes for sugar transporters playing a key role in the overall plant responses. Gene set and pathway enrichment analyses highlight the role of sucrose and starch metabolism in symptom appearance. Within this gene category, we identified which HLB-regulated genes (glucose-phosphate-transporter, invertase, starch-related genes) seem to determine the source-sink relationship disruption. In infected leaves, transcriptomic changes were observed for light reactions genes (downregulation), sucrose metabolism (upregulation), and starch biosynthesis (upregulation). In parallel, symptomatic fruits over-expressed genes involved in photosynthesis, sucrose, and raffinose metabolism, and downregulated starch biosynthesis. We visualized gene networks between tissues inducing a source-sink shift. HLB alters the hormone crosstalk, resulting in weak and ineffective tissue-specific plant immune responses. Systemic acquired responses were inadequately activated in young leaves, where infections typically occur. Unexpectedly, expression of WRKYs (including WRKY70) was higher in fruits than in leaves. Based on these results, we propose short-term therapeutic strategies for infected trees to potentiate key host response genes.

Quantification of *Candidatus Liberibacter asiaticus* in plant extracts—from Ct to biologically meaningful units

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Outcome Group 1

Candidatus Liberibacter asiaticus (CLas) is a phloem-limited bacterium associated with the citrus disease Huanglongbing (HLB), regarded to be the most devastating disease of citrus. HLB has been confirmed in all citrus producing counties in Florida and threatens viability of the citrus industry. In the early stages of HLB, diagnosis is difficult because disease symptoms are easily confused with other disorders, especially micronutrient deficiencies. Initially, quantitative real time PCR (qPCR) of CLas 16S rDNA was used to confirm CLas infection in citrus trees with suspect HLB symptoms. Confirmatory testing is a dichotomous no/yes assay. Typically, qPCR results are expressed simply as crossing threshold (Ct) and values below an arbitrary Ct are interpreted as CLas positive. However, Ct values alone have no biological context and variability in qPCR protocols among laboratories makes the reliability of arbitrary Ct values for diagnostics questionable. Increasingly, qPCR is being used as a quantitative assay in experiments related to host –pathogen interactions and in attempts to quantify resistance to CLas. Conversion of Ct values to biological units (i.e. amount of CLas per mass tissue) provides biological context and allows for meaningful comparisons among laboratories. We developed a standard curve to convert Ct to biological units (CLas genomes) and have determined that the working range for qPCR quantification of CLas is 0 to 7 logs and that qPCR can detect a single copy of CLas 16S rDNA, indicating that it is not possible to develop an assay with greater sensitivity than qPCR for CLas detection. Conversion of CLas genomes to mass reveals that Ct values less than 18 are nonsensical because the amount of CLas would exceed > 1% of total DNA suggesting that the upper limit of CLas titer in plant tissues is 10^7 to 10^8 CLas genomes·g⁻¹. Using our standard curve to convert Ct values to CLas genomes ·g⁻¹ tissue for 20,000 field samples collected by commercial scouts reveals that: 1) the majority of samples were CLas-negative (Ct > than 38.5); 2) CLas-infected, asymptomatic leaves have CLas titers of 10^2 to 10^4 genomes g⁻¹ tissue (Ct 38-30); 3) the titer of CLas in HLB-symptomatic tissues is 10^6 to 10^7 genomes· g⁻¹ tissues (Ct 24-19) and no samples exceed 10^8 CLas genomes g tissue (Ct < 18). These results confirm the value of the standard curve method for conversion of Ct to CLas genomes and provide insights into the distribution of CLas titers in infected trees.

Detection of Liberibacter infection in citrus trees through ¹H-NMR-based metabolomics of fruit and leaves

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Outcome Group 1

Infection of citrus trees by *Candidatus Liberibacter asiaticus* (Las) is devastating, leading to poor fruit taste, reduced plant vigor and productivity, and ultimately death of the tree. One of major difficulties in combating the disease is the fact that it can take up to two years following initial infection before outward symptoms become apparent. At present, Las infection is confirmed primarily by using polymerase chain reaction (PCR). However, the detection of bacteria in plant tissues by PCR has limited effectiveness in asymptomatic trees or during latent infection due to low bacterial titers within the infected plant. The existing knowledge gap in our understanding of Las and its pathogenesis leading to HLB has stymied developments of treatments and methods to mitigate the pathogen's influence. Plant metabolomics, by measuring metabolites present in a chosen tissue or fluid at a specific point in time, provides scientists with an important tool for understanding how plant metabolism responds to various environmental conditions. We have studied both fruit and leafy tissue from citrus trees using NMR-based metabolomics and observed concentration differences between samples from Las⁻ and Las⁺ trees. Some of these changes may be attributed to the effect of Las infection on plant defense mechanisms. Our studies are important for understanding how the Las bacterium may be able to evade plant defenses and may be helpful in designing new treatments for Las infection.

Physiological and proteomic characterizations of pre-symptomatic and symptomatic grapefruit (*Citrus paradisi*) plants infected with ‘*Candidatus Liberibacter asiaticus*’

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Outcome Group 3

Huanglongbing (HLB) is a highly destructive citrus disease which threatens citrus production worldwide. Among three ‘*Candidatus Liberibacter*’ species associated with HLB, ‘*Ca. Liberibacter asiaticus*’ (Las) is the most prevalent worldwide. To better understand the physiological and molecular processes involved in host responses to Las infection, proteomic analyses via 2-DE and mass spectrometry, as well as ICP spectroscopy analysis, were employed to elucidate the global protein expression profiles and nutrient concentrations in leaves of grapefruit plants that were infected with Las at pre-symptomatic or symptomatic stages of HLB. A Las-mediated down-regulation of 71 protein spots that matched to 56 proteins including those associated with photosynthesis, protein synthesis, and metabolism was correlated with significant reductions in the concentrations of Ca, Mg, Fe, Zn, Mn, and Cu, especially in symptomatic plants. Interestingly, a Las-mediated up-regulation of 26 protein spots that matched to 13 proteins including those associated with pathogen response, redox-homeostasis and starch anabolism was correlated with an increase in K concentration in pre-symptomatic and symptomatic plants. Since starch synthase requires K for activation, this study is the first to highlight a coordinated accumulation of granule-bound starch synthase and K in Las-infected plants. Those proteins that are up regulated specifically in response to Las infection could be useful biomarkers to develop a host-based diagnostic tools for early detection of HLB-affected citrus plants.

Model systems for a chemical genomics approach to HLB

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Outcome Group 3

To better understand plant-pathogen interactions in Huanglongbing disease and develop control strategies we investigated a novel approach known as chemical genomics with Tomato “Psyllid Yellows”, caused by *Candidatus Liberibacter psyllaerous* (CLps), as model of HLB. Chemical genomics involves three key stages starting with designing and performing high-throughput chemical screening, identifying chemicals inducing desired effects and dissecting the genetic targets of the candidate chemical. This approach has been used in various plant development and defense studies with *Arabidopsis thaliana*. We are investigating the suitability of Arabidopsis and tomato for studying plant-pathogen interactions in psyllid yellows and feasibility in performing high-throughput chemical screening. Initial experiments showed that Arabidopsis plants could be infected with CLps by tomato psyllid nymphs. To design a high-throughput chemical screen, we tested psyllid transmission of CLps to Arabidopsis and tomato in soilless media (solid, liquid, and hydroponic) but infection rates were too low. We tested various Arabidopsis genotypes to identify CLps -susceptible and CLps-resistant genotypes, but no ecotypes were completely resistant. In addition to lack of a CLps-symptomatic phenotype, repeated experiments revealed inconsistency in obtaining CLps-positive plants with Arabidopsis. A detached leaf assay with tomato produced consistently high numbers of CLps positive tissues such as petioles (80-85%). The detached leaf assay appears suitable for high-throughput chemical screening. To develop methods to test responses to candidate chemicals in citrus, we are testing gene expression of sweet orange seedlings following exposure to four chemicals known to induce defense responses in other plants.

A novel *Aromatic* compound acts synergistically with a naturally occurring monoterpene to elicit strong behavioral responses in Asian citrus psyllid

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Outcome Group 2

Inscent, Inc. has developed methodologies for rapidly screening potential ligands of chemosensory proteins (CSPs) isolated from the antennae of target insects. These novel ligands, referred to as *Aromatics*, mimic naturally-occurring odorants and may function as super-stimuli because of their strong affinity to chemosensory proteins. CSPs from the Asian citrus psyllid (ACP) antennae were identified based on their reactivity to Petitgrain oil. Several ligands were identified that bound these CSPs; they were then subjected to two behavioral assays to measure their biological activity. One assay measured probing frequency into a line of emulsified wax (SPLAT®, ISCA Technologies) containing the ligand. The second measured the retention time of ACP in a perforated foil cup that emitted an airstream carrying the ligand. One ligand, nicknamed 'Titan', induced significantly more probing than did limonene, a common citrus volatile and known ACP attractant. A mixture of Titan and limonene induced a probing level that was twice as high as limonene alone and significantly higher than Titan alone. This result showed that, when mixed with certain terpenes, Titan had a synergistic effect on psyllid response. In the olfactometer, there was no difference between ACP response to Titan and to the odor of orange jasmine flush, demonstrating that ACP could detect Titan via olfaction alone. As in the probing test, a mixture of limonene and Titan was also significantly more stimulatory than limonene alone. These results indicate that Titan is highly biological active, and that it could potentially be used to enhance the attractiveness of citrus volatiles.

Evaluation of *Hirsutella citriformis* Speare Mexican isolates against *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae) in laboratory

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Outcome Group 2

Diaphorina citri Kuwayama (Hemiptera: Psyllidae), the vector of the pathogen causing Huanglongbing, has been found infected by the entomopathogenic fungus *Hirsutella citriformis* Speare in at least 10 different geographic regions of the Mexican citrus industry. As alternative control methods are needed for the management of the vector under different scenarios of Mexico, this study evaluated the potential of two *H. citriformis* isolates against adults of *D. citri*. The experiments were conducted under controlled laboratory conditions ($25\pm 2^{\circ}\text{C}$, $76\pm 4\%$ RH and 16:8 h L:D). The isolates in evaluation were obtained from *H. citriformis* infected adults of *D. citri* found in orange groves located in Huimanguillo, Tabasco, and Xolol, San Luis Potosí, Mex. Insects were inoculated by contact with sporulated cultures of the isolates; afterwards, they were placed in boxes with Valencia orange leaves for feeding. For each *H. citriformis* isolate, 15 adults of *D. citri* received the spores of the fungus. Each treatment had eight replications. The results showed that mortality of the psyllids by the fungus began six days after inoculation; occurrence of the first *H. citriformis* sennemata in the *D. citri* specimens was observed 10 days after inoculation. The final record of survivorship was performed 27 days after the beginning of the experiments; the mean rate of mortality was 98 and 70% for the Tabasco, and San Luis Potosi *H. citriformis* isolates, respectively. In the control the recorded mortality was 12%. The results have implications for the use of *H. citriformis* for the management of *D. citri* in Mexico.

Success of the Citrus Health Management Area (CHMA) program in Florida

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Outcome Group 2

Following the discovery of citrus greening disease in Florida in 2005, psyllid control has become the primary pest management consideration of Florida growers. Experience has shown that while it is relatively easy to kill psyllids using commercially available insecticides, actually gaining control of psyllid populations is much more difficult. This is due to the mobility of the psyllid, coupled with the short residual activity provided by insecticides under field conditions. As a result, a recently sprayed grove may become recolonized in a matter of days as psyllids migrate in from surrounding unsprayed citrus groves. In order to enhance psyllid control programs and subsequently reduce the rate of spread of greening disease, Citrus Health Management Areas (CHMAs) were established throughout the state. The goal of the CHMA program is to coordinate the timing of insecticide applications for psyllid control by all growers within a CHMA. Such coordinated efforts will reduce “psyllid swapping” between groves and potentially minimize the need for frequent reapplication of pesticides to maintain psyllids below detectable levels. A secondary goal of the CHMA program is to manage pesticide resistance development in psyllid populations. Thus, growers within a CHMA rotate (collectively) between pesticide modes of action from one coordinated spray to the next. The CHMA program was officially kicked off in 2010 with 7 CHMAs formalized in areas of the state where grower interest was sufficient to attempt such a voluntary program. Based on the success achieved in these original CHMAs, grower interest and willingness to participate in the CHMA program has increased dramatically. To date, there are 38 CHMAs encompassing 486,079 commercial grove acres in Florida. As grower participation in the CHMA program has increased, there has been a corresponding drop in psyllid populations statewide. Through a cooperative ACP monitoring effort between FDACS-DPI and USDA-APHIS-PPQ, 6,000 citrus grove blocks are surveyed every three weeks and ACP counts are summarized for growers and CHMA coordinators. Thus far in 2012, the data indicate that psyllid populations have declined nearly 70% when compared to counts made during the same time period in 2011. Additional details on the function and success of the CHMA program in Florida are discussed.

Citrus Greening-HLB Genome Resources Group: A bioinformatics resource for diverse projects related to the biology and diagnosis of citrus greening/HLB

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Outcome Group 2

The CG-HLB Genome Resources Group serves as a bioinformatics resource for diverse projects related to the biology and diagnosis of citrus greening/HLB. Current projects include the identification of bacterial endosymbionts in the *Diaphorina citri* (Asian citrus psyllid) metagenome. Mapping sequence reads to reference genomes shows evidence for *Wolbachia* and an enterobacterial endosymbiont related to *Salmonella* in the metagenome. A draft genome sequence has been produced for the *Wolbachia* endosymbiont of *D. citri* (wDi) and the gene content analyzed for the presence of ankyrin domain and other predicted proteins potentially impacting psyllid biology. Phylogenetic analysis of individual *Wolbachia* gene sequences supports the non-Chinese origin of the Florida *D. citri* isolate being sequenced. Annotation of the psyllid genome itself is ongoing. We have additionally provided bioinformatic assistance to the Angela Douglas lab (Cornell) in extraction of individual transporter sequences from the ACP transcriptome for use in the development of RNAi-based insect control strategies. Genome comparison of *Ca. Liberibacter* isolates is being conducted to explore the sources of biological differences and to identify sites useful for diagnostic purposes. Genome characterization and comparison of publically available *Liberibacter* genome sequences (*Ca. L. asiaticus* psy62 and *Ca. L. solanacearum* ZC-1) is available at the CG-HLB Genome Resources website (<http://citrusgreening.org/>). Future plans involve development of a searchable *Liberibacter* diagnostic sequence database using the over 1700 publically available *Ca. Liberibacter* gene sequences.

Significant and substantial effects of good horticultural management on yield of Huanglongbing-affected orange groves in Florida

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Outcome Group 2

By some estimates, up to 40% of citrus trees in Florida are affected by Huanglongbing (HLB). Not only is it unfeasible to eradicate the disease by removing the HLB+ trees, but HLB+ trees must be kept productive to sustain the industry and generate revenue to support the research being conducted to find permanent solutions. We have been evaluating the effects of comprehensive grove management programs, including intensive psyllid control, good fertilization practices and irrigation management, on yield and fruit quality in commercial and research groves. In a 180 acre research grove, with >80% HLB infection, per acre yield of 'Hamlin' trees has recovered to 79% of pre-HLB yield, and yield of 'Valencia' trees has recovered to 100% of pre-HLB yield following 3-years of intensive management. Under commercial grove conditions with virtually 100% HLB infection, 'Hamlin' trees yielded 600 boxes/acre and 'Valencia' trees 514 boxes/acre for the 2011-12 harvest season – 6 years after HLB was first found in the grove. Perhaps more importantly, a block of young 'Valencia' trees (~40 acres) planted in 1999 yielded 83 boxes/acre in 2005-06 when HLB was first detected, and is now producing 242 boxes/acre in 2011-12, almost as high as the state average of 287 boxes/acre. Fruit quality in both groves remains excellent. The data we have collected support the hypothesis that HLB is not a lethal disease but instead causes rapid tree decline, which can be successfully reversed with good management, producing yields of quality fruit equal to or significantly greater than historical yields.

Vector control and foliar nutrition for management of Huanglongbing in Florida citrus

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Outcome Group 1

Vector control and foliar nutrition are widely employed in Florida citrus to respectively slow the spread of HLB and mitigate effects of the disease on citrus trees. A replicated field study was conducted in a 13-acre commercial block of young 'Valencia' through four harvests employing a factorial design to evaluate individual and compound effects of a popular nutrient/SARs program and threshold-based vector management. Asian citrus psyllid populations were maintained at contrasting levels in insecticide-treated and untreated plots despite proximity. Nevertheless, incidence of HLB, estimated by PCR at near 30% at the beginning of the study, rose to over 90% early in the third year without significant contribution from vector control. However, yields were significantly improved all four years by the combined effects of nutrition and vector control, with a significant contribution from nutrition in the fourth year when production in the combined treatment approached the pre-HLB regional average. Economic analysis showed that, while the Insecticide + Nutritional treatment produced the highest gain in production, it would not have been profitable at current juice prices. Fine tuning by reducing costs of the nutrient package and/or the insecticide regime could have made the program profitable, as could higher margins of a fresh fruit market. Further research is necessary to establish economic thresholds for both insecticide and nutrient application under different market and environmental conditions.

Under severe citrus canker and HLB pressure, Triumph and Jackson perform better than Flame and Marsh grapefruit

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Outcome Group 3

Citrus Canker (CC) and Huanglongbing (HLB) threaten the viability of Florida grapefruit production. ‘Triumph’ (T), reportedly a grapefruit/sweet orange hybrid, is similar to seedy white grapefruit with earlier maturity and lower bitterness. ‘Jackson’ (J) is a low-seeded budsport of ‘Triumph’. Tree health and productivity of T, J, and standard cultivars ‘Marsh’ (M) and ‘Flame’ (F) planted in a replicated field trial were assessed for 3 years, in a site with endemic CC and HLB. In each year overall tree health of T/J was significantly greater than F/M. Severity of CC was significantly less on T/J than on F/M, while foliar HLB symptoms and titers of *Liberibacter asiaticus* were similar among cultivars. F/M were almost completely defoliated in some years while T/J had normal canopy density. Cumulative numbers of fruit/tree were greater for T/J (255/220) than for F/M (29/66). Tree height of T/J was greater than F/M. Canopy volume was greater in T/J than F/M in some years, but trunk cross sectional area was not different. Cumulative percent fruit drop was greater in F/M than T/J (F=50; M=53; T=15; J=14). Fruit quality assessments were made each cropping season. T/J fruit met commercial maturity standards whereas F/M fruit did not due to low total soluble solids and low Brix/acid ratios. In 2011/2012 many F/M were small and/or misshapen while T/J fruit were of normal size and shape. These results suggest that T/J or other grapefruit-like cultivars may be viable alternatives to standard grapefruit cultivars in the presence of severe CC and HLB.

Effect of entomopathogenic fungi on *Diaphorina citri* (Hemiptera: Psyllidae) and its predator *Hippodamia convergens* (Coleoptera: Coccinellidae)

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Outcome Group 2

The strategy for the management of Huanglongbing in the citrus industry of Mexico, includes the population control of the vector, *Diaphorina citri* (Hemiptera: Psyllidae). In order to contribute in the practice of rational control methods, this study evaluated diverse isolates of the fungi *Isaria fumosorosea* and *Metarhizium anisopliae* against *D. citri*. We also documented the effects in its predator *Hippodamia convergens* (Coleoptera: Coccinellidae). We evaluated in laboratory four isolates of *I. fumosorosea* and eight of *M. anisopliae* against adults of *D. citri* and *H. convergens* using 1×10^8 spores/ml in 0.03% Tween 80 + Citrus King oil. The control treatment was sprayed with the mixture of Tween + oil. After the application of the fungi, *D. citri* specimens were kept in plastic cages with orange leaves; *H. convergens* adults were maintained in Petri dishes and fed with *Sitotroga cerealella* eggs, an artificial diet, and water+honey. All the insects were upheld under lab conditions ($25 \pm 2^\circ\text{C}$, $70 \pm 4\%$ RH and 16:8 h L:D). The results showed that mean mortality of *D. citri* caused by *I. fumosorosea* ranged 31% (the control)- 83% (CIS45 isolate) (n=10). In *M. anisopliae*, the lowest mean mortality was recorded in the control (19%); meanwhile, high mortality was observed for the C122 and C127 isolates (average mortality= 88 and 79%, respectively). These isolates of *M. anisopliae* against *D. citri* caused 66-69% mean mortality in *H. convergens* (n=4). The *I. fumosorosea* CIS45 isolate produced 24% mortality in the predator. The study has implications for the selection and use of entomopathogenic fungi in the biological control of *D. citri*.

Response of government and the citrus industry to the discovery of Asian citrus psyllid in Arizona

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Outcome Group 2

In October, 2009, about three months after the first find of Asian Citrus Psyllid (ACP) in San Luis Rio Colorado Sonora, a colony of ACP was found just across the border in San Luis Arizona. Since then, twelve additional sites have been found in Arizona, all except two in Yuma County. Less than 50 individual ACP have been found since 2009 and all have been eradicated. No ACP found in Arizona has yet tested positive for HLB. As of now, much of southwest Arizona is under federal quarantine for ACP and trees and fruit that move out of the quarantine area require special treatment. The response of the Arizona Department of Agriculture to the discovery of ACP has been to increase trapping and eradication activities using funds received from the Federal and State Government. The University of Arizona and the citrus industry have responded by establishing screen houses to produce trees that can provide disease-free budwood. The industry has also developed a plan to establish an area-wide spray program if eradication efforts are not successful. Extension and outreach efforts have been directed toward the industry and the homeowner. The location of ACP finds in Arizona suggests that both Mexican ACP populations near the border and the transport of citrus fruit from the interior of Mexico lead to the establishment of the insect in Arizona. The small numbers of ACP found in Arizona, in contrast with populations found in coastal California, suggest that ACP populations may be adversely affected by the arid climate of the region, and that timely detection and eradication efforts are the keys to controlling spread of the ACP in arid regions.